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Naturally occurring Toll-like receptor 11 (*TLR11*) and Toll-like receptor 12 (*TLR12*) polymorphisms are not associated with *Toxoplasma gondii* infection in wild wood mice

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Running headline: *TLR* variation and *Toxoplasma gondii* infection

Abstract

Toxoplasma gondii is a highly successful parasite with a worldwide prevalence. Small rodents are the main intermediate hosts, and there is growing evidence that *T. gondii* modifies their behavior. Chronically infected rodents show impaired learning capacity, enhanced activity, and, most importantly, a reduction of the innate fear towards cat odour. This modification of host behavior ensures a successful transmission of *T. gondii* from rodents to felids, the definitive hosts of the parasite. Given the negative fitness consequences of this behavioral manipulation, as well as an increased mortality during the acute phase of infection, we expect rodents to evolve potent resistance mechanisms that prevent or control infection. Indeed, studies in laboratory mice have identified candidate genes for *T. gondii* resistance. Of particular importance appear to be the innate immune receptors Toll-like receptor 11 (TLR11) and Toll-like receptor 12 (TLR12), which recognize *T. gondii* profilin and initiate immune responses against the parasite.

Here we analyse the genetic diversity of *TLR11* and *TLR12* in a natural population of wood mice (*Apodemus sylvaticus*), and test for associations between *TLR11* and *TLR12* polymorphisms and *T. gondii* infection, as well as for epistatic interactions between *TLR11* and *TLR12* on infection status. We found that both *TLR11* and *TLR12* were polymorphic in wood mice, with four and nine amino acid haplotypes, respectively. However, we found no evidence that *TLR11* or *TLR12* genotypes or haplotypes were significantly associated with *Toxoplasma* infection. Despite the importance of TLR11 and TLR12 in *T. gondii* recognition and immune defence initiation, naturally occurring polymorphisms at *TLR11* and *TLR12* thus appear to play a minor role in mediating qualitative resistance to *T. gondii* in natural host populations of *A. sylvaticus*. This highlights the importance of assessing the role of candidate genes for parasite resistance identified in a laboratory setting in an

ecologically meaningful context to quantify their role in mediating host-parasite interactions in the wild.

Keywords: *Toxoplasma gondii*, Toll-like receptors, immunogenetics, parasite resistance, *Apodemus sylvaticus*, zoonotic disease

1. Introduction

Individuals differ substantially in their susceptibility to parasites and pathogens, and this variation has at least partly a heritable basis (Kaslow et al., 2008). Genetic diversity in host resistance can be maintained by several non-mutually exclusive mechanisms, including negative frequency-dependent selection, overdominance, or temporal and spatial variation in the composition of the parasite community (Apanius et al., 1997; Hedrick, 2002; Woolhouse et al., 2002). Due to their intimate interaction with the parasite, genes involved in parasite recognition, rather than immune signalling or effector genes, are predicted to be main targets of parasite-mediated directional selection (Tiffin and Moeller, 2006; Sackton et al., 2007; but see Fumagalli et al., 2009; Turner et al., 2012).

Biomedical research has identified candidate genes involved in host defence against a large number of disease causing agents (Hill, 2001). A common approach is to use knockout mice, in which the target gene has been made inoperative, to demonstrate an increased susceptibility to experimental infections (Li et al., 1999; Alexopoulou et al., 2002). Whereas such an approach can provide important insights into parasite infection and host defence pathways, it is agnostic about the level of naturally occurring polymorphisms (laboratory mice are usually highly inbred), or the role of the identified gene in coevolutionary processes in natural populations. Indeed, parasite-mediated selection can only act if there is variation in host defence traits. Thus, it remains largely unclear if candidate genes for parasite resistance identified in a laboratory setting mediate variation in host resistance under ecologically meaningful conditions, especially in non-human vertebrates.

Also, there is accumulating evidence that environmental factors play a crucial role in shaping immune system functioning, and thus the way hosts interact with their parasites (Abolins et al., 2011; Boysen et al., 2011). Therefore, we may gain

important insights into the evolution and function of the vertebrate immune system by studying patterns of infection in natural host-parasite systems (Pedersen and Babayan, 2011; Maizels and Nussey, 2013; Turner and Paterson, 2013). This could prove to be particularly fruitful for our understanding of defence strategies against zoonotic diseases, given the long co-evolutionary history of wildlife with the disease causing agents.

Toxoplasma gondii is an apicomplexan parasite and the causative agent of human toxoplasmosis. It has a worldwide distribution and a broad range of intermediate hosts, but with only felids as definitive hosts (Tenter et al., 2000). Rodents play a particularly important role as intermediate hosts, because they are a prey for cats (Tenter et al., 2000). Furthermore, vertical transmission in rodents seems to contribute to the maintenance of *Toxoplasma* in areas with low cat density (Hide et al., 2009; Thomasson et al., 2011).

In most host species, infections with *T. gondii* are asymptomatic (Yarovinsky et al. 2008). However, in mice *T. gondii* appears to be more virulent, with a high mortality rate during the acute phase of infection (Zenner et al., 1998). Additionally, several studies have documented an alteration of behavior in chronically infected hosts (Webster and McConkey, 2010). Studies in laboratory mice, for example, have shown an impaired learning capacity (Hodkova et al., 2007; Webster, 2007), enhanced activity patterns (Hodkova et al., 2007; but see Hrdá et al., 2000) , and, most importantly, a reduction in the natural fear towards cat odour (Vyas et al., 2007; Xiao et al., 2012; but see Worth et al., 2013) in *T. gondii* infected rodents. The latter is likely to increase the host's predation risk in the wild. Given these negative fitness consequences of infection, we would expect rodents to evolve potent resistance mechanisms that prevent or control *T. gondii* infections.

Pattern-recognition receptors (PRRs) belong to the innate branch of the vertebrate immune system and play an important role in the first line of defence against invading parasites (Akira et al., 2006). One important group of PRRs is the Toll-like receptor (TLR) family, which has diversified into several different TLRs in vertebrates, each recognizing specific pathogen structures (Roach et al., 2005). Studies have found a pronounced susceptibility to *T. gondii* in laboratory mice lacking the TLR adaptor protein MyD88, suggesting an involvement of TLRs in *T. gondii* defence (Scanga et al., 2002). Subsequent knock-out studies revealed that it is in particular TLR11, which plays an important role in *T. gondii* resistance. TLR11-deficient mice are highly susceptible to *Toxoplasma* compared to wild-type mice (reviewed in Pifer and Yarovinsky, 2011; Yarovinsky, 2014). Moreover, it was found that the recently characterised receptor TLR12 also influences resistance to *T. gondii* in laboratory mice (Koblansky et al., 2013), either on its own (Koblansky et al., 2013) or by forming heterodimers with TLR11 (Andrade et al., 2013; Raetz et al., 2013). Indeed, mortality rates of TLR12-deficient mice even exceeded those of TLR11-deficient mice during the acute phase of *T. gondii* infection (Koblansky et al., 2013). The ligand recognised by TLR11 and TLR12 is profilin (Yarovinsky et al., 2005; Kucera et al., 2010), which plays an important role in the motility, host cell invasion, and virulence of *Toxoplasma* (Plattner et al., 2008). Interestingly, both *TLR11* and *TLR12* are expressed exclusively in rodents, and whereas *TLR11* exists as a pseudogene in humans, *TLR12* is completely absent in the human genome (Roach et al., 2005). Given the important role of TLR11 and TLR12 in *Toxoplasma* recognition, we would predict polymorphisms at *TLR11* and / or *TLR12* ligand-binding sites to affect *Toxoplasma* susceptibility in the wild.

Previous studies in humans and domestic animals have found that *TLR* polymorphisms affect susceptibility to a wide range of parasites and pathogens (e.g.

Brightbill et al., 1999; Texereau et al., 2005; Garantziotis et al., 2008). Yet, very few studies have tested how naturally occurring polymorphisms at *TLRs* influence disease susceptibility in wildlife (but see e.g. Jackson et al., 2009; Turner et al., 2011; Tschirren et al., 2013). Here we quantified *TLR11* and *TLR12* genetic diversity and tested for associations between *TLR* polymorphisms and *T. gondii* infection in a natural population of wood mice (*Apodemus sylvaticus*).

2. Material and Methods

2.1 Sample collection and detection of *Toxoplasma gondii* infection

Wood mice (*Apodemus sylvaticus*) are one of the main intermediate hosts of *T. gondii* in Europe and infection prevalence can reach high levels within populations (Thomasson et al., 2011). For this study, wood mice were trapped using Longworth small mammal traps at four sites (Tarn Wood 54°06'03.3"N, 002°09'44.9"W, Spiggot Hill 54°05'72.9"N, 002°10' 43.1"W; Ha Mire Plantation 54°05'64.5"N, 002°09' 53.7"W and Tarn Fen 54°06'00.0"N, 002°10'43.4"W) within the boundaries of the Malham Tarn Nature Reserve in North Yorkshire, UK between October 2009 and October 2011 under a permit granted from the National Trust (see Boyce et al., 2012; Boyce et al., 2013 for details). The majority of mice were caught during the summer and autumn. Animals were euthanized, their body mass and body length (measured from nose to anus using a measuring board) was measured and they were dissected. The body mass of four individuals was not available. We calculated the scaled mass index as a measure of body condition (Peig & Green, 2009). DNA was extracted from brain tissue using a phenol/chloroform protocol and the presence of *T. gondii* was detected by PCR amplification of the *Surface Antigen Gene 1* (*SAG1*) as described in Thomasson et al. (2011). Infection was confirmed using a further panel of *Toxoplasma*-specific PCR diagnostic genes – *SAG2*, *SAG3* and *GRA6* (Bajnok et al.,

submitted). Ethical approval for this work was given by the College of Science and Technology Research Ethics Panel, University of Salford (Reference: CST 12/36).

2.2 *TLR11* and *TLR12* genotyping

TLR11 and *TLR12* sites involved in pathogen recognition, so called leucine-rich repeat (LRR) motives, were predicted using the TollML database (Gong et al., 2010). A 979 bp long fragment of *TLR11* and a 862 bp long fragment of *TLR12*, containing most of these LRR regions, was sequenced in 120 wood mice. Primers (*TLR11*ASF: 5'-CAG GGC ATG GTT CCT ACT CT-3'; *TLR11*ASR: 5'-TCT TGG GAC CCT GAA GTT GT-3' and *TLR12*ASF: 5'-CCT GAT GCC TTY RGT GAC CT-3'; *TLR12*ASR: 5'-GCC GYT TAT RGT CAA GGT CT-3') were designed based on the house mouse (GenBank accession numbers NM_205819.3 and AY510705.1) and rat (GeneBank accession numbers NM_001144779.2 and NM_001108682.1) *TLR11* and *TLR12* sequences using the program Primer3 (Rozen & Skaletsky 2000). Clean wood mouse sequences were obtained using these primers and a NCBI GenBank Blast search confirmed the amplification of *TLR11* and *TLR12*, respectively. Polymerase chain reactions (PCR) were carried out on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Amplifications were performed in a total volume of 10 µl containing 0.2 µl JumpStart Taq DNA Polymerase (Sigma-Aldrich), 300 nM of each primer and 1 µl DNA. The PCR protocol consisted of an initial denaturation step at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 90 sec, with a final elongation step at 72°C for 10 min. PCR products were purified and sequenced in both directions on an ABI Prism 3730 capillary sequencer (Applied Biosystems) using Big Dye terminator version 3.1 chemistry (Applied Biosystems). All *TLR11* and *TLR12* raw sequences were trimmed, processed and aligned in Geneious 5.6.5 (Drummond et al., 2009). Polymorphisms were detected by eye and haplotypes were

reconstructed in the program PHASE 2.1 (Stephens et al., 2001) using default settings. *TLR11* (accession numbers: KJ612408- KJ612411) and *TLR12* (accession numbers: KJ612412 - KJ612420) haplotypes were submitted to GenBank.

2.3 Statistical analyses

The genealogy of *TLR11* and *TLR12* haplotypes was inferred with the program TCS (Clement et al., 2000), taking both synonymous and nonsynonymous mutation into account.

We tested if *T. gondii* infection was associated with *TLR11* and / or *TLR12* genotypes (i.e. haplotype combinations), and if infection status differed between individuals that were *TLR11* or *TLR12* homozygous (twice the same haplotype) vs. heterozygous (two different haplotypes) using generalized linear models with a binomial error structure. We tested if epistatic interactions between *TLR11* and *TLR12* influenced infection status by fitting the interaction term between *TLR11* and *TLR12* genotypes in the model. Furthermore, we tested if *T. gondii* infection status is associated with certain *TLR11* or *TLR12* haplotypes, again using generalized linear models with a binomial error structure. Sex, body condition and sampling location were included as factors in the analyses to account for potential sex-specific or condition-related differences in infection patterns or potential differences in *T. gondii* prevalence among sampling locations. Genotypes and haplotypes that occurred at frequencies < 5% were pooled in a 'rare' category for the analyses. The significance of the different variables was determined by comparing two nested models, one with and one without the factor of interest, using likelihood-ratio tests. Statistical analyses were performed in R 2.14.2 (R Development Core Team, 2011).

3. Results

We detected six *TLR11* haplotypes in the studied wood mouse population, of which four differed at the amino acid level. *TLR11* haplotypes 1 and 2 occurred at high frequencies (*TLR11*_{H1}: 31.9% and *TLR11*_{H2}: 65.5%), whereas haplotypes *TLR11*_{H3} and *TLR11*_{H4} were found in only five and one individual, respectively (Fig. 1). For *TLR12*, we observed 13 haplotypes of which nine differed at the amino acid level. *TLR12*_{H1} was the most common haplotype (frequency: 77.9%). Haplotypes *TLR12*_{H2} and *TLR12*_{H6} occurred at frequencies of 5.8% and 10.8%, respectively. All other haplotypes were rare (frequency: 0.4 – 1.7%, Fig. 1). The reconstructed genealogies of *TLR11* and *TLR12* haplotypes are shown in Figure 1.

Toxoplasma gondii was detected in 36% of the analysed wood mice (N = 120). Infected animals tended to be lighter ($\chi^2 = 3.263$, $DF = 1$, $P = 0.071$) and in worse condition ($\chi^2 = 3.071$, $DF = 1$, $P = 0.080$), but there was no association between body size and infection status ($\chi^2 = 0.083$, $DF = 1$, $P = 0.773$). There was a tendency for a higher infection rate in females ($\chi^2 = 2.950$, $DF = 1$, $P = 0.089$).

Toxoplasma infection status was not significantly associated with *TLR11* ($\chi^2 = 3.541$, $DF = 3$, $P = 0.315$; Table 1) or *TLR12* genotype ($\chi^2 = 4.264$, $DF = 3$, $P = 0.234$; Table 1), and there was no significant interaction effect between *TLR11* and *TLR12* genotype on *T. gondii* infection ($\chi^2 = 5.028$, $DF = 8$, $P = 0.755$). Furthermore, there was no significant association between *TLR11* (all $P > 0.283$) or *TLR12* haplotypes (all $P > 0.090$) and *Toxoplasma* infection. Finally, there was no relationship between *TLR11* ($\chi^2 = 0.330$, $DF = 1$, $P = 0.566$) or *TLR12* heterozygosity ($\chi^2 = 0.964$, $DF = 1$, $P = 0.326$) and *T. gondii* infection status.

4. Discussion

Laboratory-based studies demonstrated that *TLR11* and *TLR12*, two innate immune receptors only expressed in rodents, play a key role in the recognition and initiation of immune responses against *T. gondii* in laboratory mice (Yarovinsky, 2014). Here we transferred these findings into an ecologically meaningful context to test, firstly if *TLR11* and / or *TLR12* are polymorphic, and secondly, if *TLR11* and / or *TLR12* variants are associated with *T. gondii* infection in *Apodemus sylvaticus*, one of the main intermediate hosts of *T. gondii* in Europe.

We observed an intermediate level of genetic diversity at *TLR11*, with four amino acid haplotypes. Two of these haplotypes were rare, whereas the other two were both common and occurred at intermediate frequencies. For *TLR12*, we found that gene diversity was higher with nine haplotypes that differed at the amino acid level, but haplotype frequencies were more skewed with one haplotype being very common. Whereas this is the first study to quantify genetic diversity at *TLR11* or *TLR12* in a natural rodent population, studies on the diversity at other *TLRs* in wildlife are starting to accumulate. *TLR2* for example is highly polymorphic in bank voles (*Myodes glareolus*), but shows low levels of diversity in yellow-necked mice (*Apodemus flavicollis*) (Tschirren et al., 2011; Tschirren et al., 2012). Furthermore, in a bottlenecked population of New Zealand robin (*Petroica australis rakiura*), seven out of nine analysed *TLRs* were polymorphic, with two to five amino acid variants each (Grueber et al., 2012). This relatively high genetic diversity observed in wildlife is in agreement with human studies (Mukherjee et al., 2009; Wlasiuk and Nachman, 2010), and shows that there is the potential for parasite-mediated selection to act on *TLRs*.

Although we observed variation at *TLR11* and *TLR12* on which *T. gondii*-mediated selection could act on, we found no evidence that *TLR11* or *TLR12* genotypes or haplotypes were significantly associated with *T. gondii* infection. Interestingly, *T. gondii* prevalence was almost twice as high among animals with a rare genotype compared to animals with a common genotype. However, given the small number of animals with a rare genotype, we can currently only speculate if this difference is due to chance, or if rare *TLR11* and *TLR12* genotypes are associated with a higher *T. gondii* susceptibility, and are rare because they have a selective disadvantage. There was no evidence for epistatic interactions between *TLR11* and *TLR12* genotypes on infection status. Also, we found no indication for a *TLR11* or *TLR12* heterozygote advantage. Although *TLR11* and *TLR12* play an important role in the recognition and control of *T. gondii* in laboratory mice (Pifer and Yarovinsky, 2011; Koblansky et al., 2013), and both are expressed exclusively in rodents (Roach et al., 2005), *TLR11* and *TLR12* genetic polymorphisms thus appear to play a minor role in mediating variation in *T. gondii* susceptibility in our study population.

This finding is in contrast to a recent study on bank voles, which demonstrated a strong association between *TLR2* haplotypes and *Borrelia afzelii* infection (Tschirren et al., 2013). As for *TLR11* / *TLR12* and *T. gondii*, this study was based on the finding that *TLR2* is essential for the recognition and initiation of immune responses against *Borrelia*, and that *TLR2*-deficient mice show a markedly increased susceptibility to *Borrelia* in the laboratory (Alexopoulou et al., 2002; Wooten et al., 2002).

The lack of an association between *TLR11* and / or *TLR12* variants and *T. gondii* infection could have a number of reasons. First, we considered only infection status (i.e. qualitative resistance), but not infection intensity (i.e. quantitative resistance) in our study. Thus, we cannot exclude the possibility that *TLR11* and / or *TLR12* polymorphisms influence quantitative resistance against *T. gondii*. Second,

the severity of behavioural manipulation by *T. gondii* appears to depend on which brain regions are affected by the parasite (Haroon et al., 2012; but see McConkey et al., 2013). A more detailed analysis of infection patterns might thus increase the power to detect potential associations with *TLR* variants. Third, *T. gondii* strains differ substantially in virulence (Grigg et al., 2001; Saeij et al., 2005), at least under laboratory conditions, and we have no information on how severe the fitness consequences of *T. gondii* infection are in our study populations. If fitness consequences are comparably mild, we would not expect *T. gondii* to exert strong selection on its host's immune system. Fourth, experimental evidence that *T. gondii* susceptibility is *TLR11*- and *TLR12*-dependent comes from studies on laboratory mice. If the function of *TLRs* differs across rodent genera, this might explain the lack of an association between *TLR11* and *TLR12* variation and *T. gondii* infection in our study. However, given the high level of structural and functional conservation of *TLRs* in vertebrates (Roach et al., 2005), this scenario seems unlikely. Finally, a potential association between *TLR* variants and *T. gondii* infection could be masked by third factors that could not be controlled for in this field study. Importantly, however, and despite all these factors that may reduce statistical power, the lack of an association between *T. gondii* infection and *TLR11* / *TLR12* variants illustrates that *Toxoplasma*-mediated selection on these immune receptors is weak at best in our study population.

In conclusion, we show that although previous studies have shown that *TLR11* and *TLR12* are essential for the recognition and initiation of immune responses against *T. gondii* in laboratory mice, *TLR11* and *TLR12* polymorphisms do not appear to mediate variation in *Toxoplasma* susceptibility in wild-living wood mice. Our results indicate that pathways essential for host defence identified in a laboratory

setting are not necessarily the main targets of parasite-mediated selection in natural host-parasite systems.

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7. Declaration of interest

Authors have no declarations of interest to report.

Figure legend

Figure 1: Haplotype network

Haplotype genealogy of A) *TLR11* and B) *TLR12* based on 120 individuals.

Displayed are all haplotypes. The circle sizes reflect the number of haplotype copies observed in the population. The same numbers (e.g. 1a, 1b) identify haplotypes that are identical at the amino acid level. Each line indicates a change at one nucleotide.

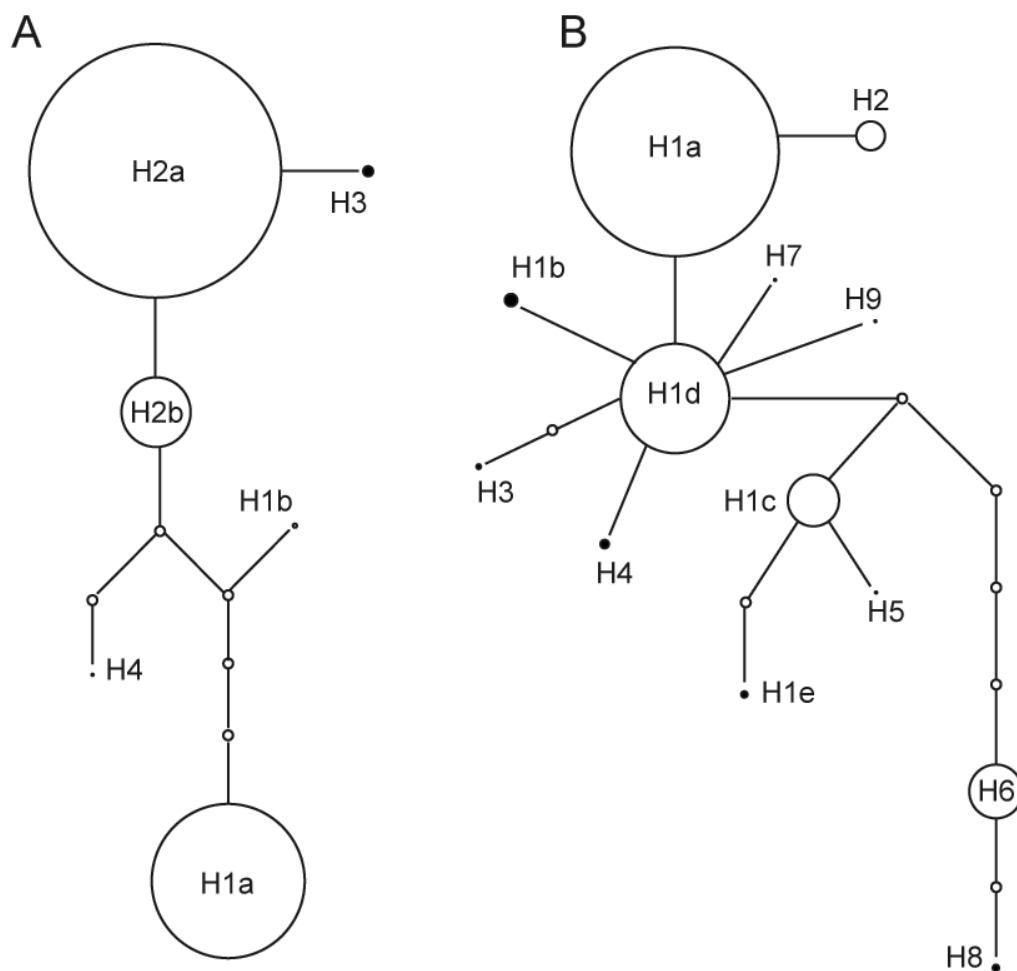


Table 1: *Toxoplasma gondii* infection across *TLR11* and *TLR12* genotypes

TLR11 and *TLR12* genotypes (synonymous haplotypes were combined), number of individuals (N), and percentage of *T. gondii*-infected individuals per genotype.

Genotypes with a frequency < 5% were combined into the category 'rare'.

<i>TLR11</i>	N	Infected [%]
<i>H1/H1</i>	18	38.9
<i>H1/H2</i>	38	31.6
<i>H2/H2</i>	58	34.5
rare	6	66.7

<i>TLR12</i>	N	Infected [%]
<i>H1/H1</i>	72	33.3
<i>H1/H2</i>	13	30.8
<i>H1/H6</i>	22	31.8
rare	13	61.5

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